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Gene Expression Profiling in Vastus Lateralis Muscle During an Acute Exacerbation of COPD

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Key Words

COPD • Exacerbation • Hospitalization • Microarray • Skeletal muscle • Wasting

Abstract

Background/aims: The molecular mechanisms leading to loss in muscle force during an acute exacerbation in COPD patients are unknown. A cross-sectional study was designed to compare the gene expression profile of the vastus lateralis muscle in patients with an acute COPD exacerbation and in stable COPD patients. Methods: Muscle biopsies were taken in 9 COPD patients with an exacerbation on day 4 of hospitalization and in 15 stable COPD patients. Microarray was performed on an UniSet Human 20K Bioarray. Results: Gene Ontology and Gene Set Enrichment Analysis of the microarray data revealed enrichment of 1) the ubiquitin-dependent protein catabolism, the induction of apoptosis and anti-apoptosis and the response to reactive oxygen species in the upregulated transcripts, and 2) the aspartate catabolism and the mitochondrial respiratory chain in the downregulated transcripts. Real Time PCR data confirmed 1) increased expression of MuRF1 and MAFbx, markers of the ubiquitin dependent catabolism pathway, and 2) decreased expression levels of COX6C, a marker of mitochondrial respiration. Conclusions: The present study suggests that multiple pathways leading to muscle atrophy and mitochondrial dysfunction are altered in the muscle during an acute exacerbation. Strategies limiting the loss of muscle function during an acute exacerbation need to be developed.

Introduction

Chronic obstructive pulmonary disease (COPD) is a slowly progressing disease characterized by airflow obstruction and chronic inflammation [1]. COPD is also associated with several systemic co-morbidities, such as osteoporosis, increased risk of cardiovascular diseases and skeletal muscle wasting [2]. Skeletal muscle weakness contributes to exercise intolerance and impaired health-related quality of life [3]. Skeletal muscle function is critical to survival and predicts the mortality risk in patients with COPD [4]. Disuse due to sedentary lifestyle [5], the use of corticosteroids [6], the presence of systemic inflammation [7] and/or oxidative stress [8] further contribute to the development and the progression of

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muscle weakness in COPD.

The mechanisms underlying the wasting process in this disease are not known. Recently, the mRNA expression profile of the quadriceps muscle in stable COPD patients identified several genes that were distinctly regulated compared to control subjects [9, 10]. Among others, genes involved in protein degradation and synthesis, energy production, muscle contraction and regeneration were selectively expressed in the quadriceps muscle of the stable COPD patients. These studies provided for the first time evidence for impaired gene expression in the muscle of stable COPD patients that may contribute to muscle dysfunction described in this population.

In COPD patients, repeated exacerbations represent a common feature of the disease. Exacerbations exert serious detrimental effects on the patient and lead to a significant increase in health care resource utilization. Exacerbations are associated with accelerated decline in lung function [11], further impairment of quality of life [12] and increased mortality risk [13]. In addition, exacerbations result in a further decline in skeletal muscle force. This loss of muscle function is rapid but recovery is slow and partial [14]. This means that in patients with frequent exacerbations, muscle weakness probably accumulates since loss of muscle function occurring during an exacerbation may take place during recovery phase.

As for stable COPD patients, the mechanisms triggering muscle weakness during exacerbations are not known. It is now accepted that during acute exacerbation, enhanced systemic inflammation is present in the lung and the systemic circulation and this may affect several systems. Local expression of inflammatory markers such as IL-6, IL-8 and TNF-α was, however, not present in the skeletal muscle of these patients [15] whereas expression levels of the anabolic markers MyoD and IGF-I were decreased in their muscle [15]. MyoD expression levels showed to be positively related to muscle force [15]. These data suggested a prominent role for local rather than systemic regulating mechanisms in the occurrence of skeletal muscle weakness during an acute COPD exacerbation.

Knowledge on the mechanisms involved in the loss of muscle function during an acute exacerbation may be helpful to develop appropriate strategies to better preserve muscle function in these patients. This is, indeed, clinically relevant knowing that recovery of muscle force is particularly slow as it does not return to values seen in stable COPD patients even three months after exacerbation. In order to unravel the potential molecular events occurring in the muscle during an acute exacerbation, a cross-sectional study was performed to compare the gene expression profile of the vastus lateralis muscle in COPD patients with an acute exacerbation and in stable COPD

	COPD with acute exacerbation	Stable COPD
N	4	5
Age (years)	71 ± 6	64 ± 10
BMI (kg/m^2)	23 ± 4	26 ± 4
CRP (mg/L)	45 ± 38*	2.7 ± 2.8
FEV_1 (%pred)	34 ± 20	48 ± 18
FEV_1/FVC (%pred)	44 ± 13	60 ± 12
PaO_2 (mmHg)	61 ± 13	66 ± 17
$PaCO_2$ (mmHg)	39 ± 6	45 ± 5
P_{Imax} (%pred)	78 ± 15	86 ± 16
QF (%pred)	46 ± 16*	90 ± 15

Table 1. Characteristics of the population included in the microarray screening. Data represent values on day 3 of hospitalization, unless specified otherwise. Values are expressed as means and standard deviation. N=number of patients per group; BMI=body mass index; CRP=C-reactive protein at hospital admission for the patients with an acute exacerbation; FEV_1=forced expiratory volume in the first second; FVC=forced vital capacity; PaO_2 and $PaCO_2$=arterial oxygen and carbon dioxide tension, P_{Imax}=maximal inspiratory mouth pressure; QF=quadriceps force; %pred=percentage of predicted value; *p<0.05.

	COPD with acute exacerbation	Stable COPD
N	9	15
Age (years)	67 ± 8	70 ± 9
BMI (kg/m^2)	24 ± 4	26 ± 4
CRP (mg/L)	33 ± 30*	10 ± 15
FEV_1 (%pred)	43 ± 17	45 ± 14
FEV_1/FVC (%pred)	47 ± 11	56 ± 15
PaO_2 (mmHg)	66 ± 13	63± 10
$PaCO_2$ (mmHg)	39 ± 6	43 ± 5
PImax (%pred)	86 ± 18	79 ± 28
QF (%pred)	61 ± 22*	78 ± 19

Table 2. Characteristics of the population included in the PCR experiment. Values are expressed as means and standard deviation. N=number of patients per group; BMI=body mass index; CRP=C-reactive protein at hospital admission for the patients with an acute exacerbation; FEV_1=forced expiratory volume in the first second; FVC=forced vital capacity; PaO_2 and $PaCO_2$=arterial oxygen and carbon dioxide tension, P_{Imax}=maximal inspiratory mouth pressure; QF=quadriceps force; %pred=percentage of predicted value; *p<0.05.

patients. We hypothetized that the investigation of the mRNA expression profile of the quadriceps muscle may be a useful screening tool to identify key pathways involved in the loss of muscle function seen in COPD patients during an acute exacerbation. These data may lead to relevant issues to prevent or minimize further deterioration of muscle function in this population with muscle weakness.

Materials and Methods

Study design

First, a microarray screening was performed on the vastus lateralis biopsy obtained from 4 COPD patients with an acute exacerbation and 5 stable COPD patients. After completing the whole data analysis of the micro-array screening, real time PCR analysis of key genes was subsequently performed on 9 COPD patients with an acute exacerbation and 13 stable COPD patients. Eight out of the 9 COPD patients with acute exacerbation and 8 out of the 15 stable COPD patients took part in a previous study [15].

Patient population

Nine male COPD patients (forced expiratory volume in the first second/forced vital capacity, $FEV_1/FVC<70\%$) hospitalized for an acute exacerbation were compared to 15 clinically stable male COPD patients visiting the outpatient clinic ($FEV_1/FVC<70\%$, no hospitalizations for acute exacerbations within 1 year before testing, no participation in training programs). They were matched for age, FEV_1 and lung function. An acute COPD exacerbation was defined as an increase in symptoms of dyspnoea, sputum volume and purulence and cough frequency for at least 48 hours. The decision for hospital admission was made by the attending chest physician present in the emergency room. Hospitalized patients received 32mg/day oral methylprednisolone for 1 week, followed by 24mg/day for 4 days and a subsequent decrease of 4mg/week. On day 3 of hospitalization, pulmonary function, maximal inspiratory mouth pressure, and quadriceps peak torque were measured in all hospitalized patients. On day 4 of hospitalization, percutaneous Bergström needle biopsies were taken from the vastus lateralis muscle. All stable COPD patients performed the aforementioned tests on 2 separate days in an outpatient setting and vastus lateralis biopsies were taken. Characteristics and physiological data of the population are summarized in table 1 for the micro-array screening and in table 2 for the real time PCR experiment. All participants gave oral and written informed consent to participate in the present study. This study was approved by the Ethics Committee of the University Hospitals Leuven.

Protocol

RNA isolation. Total RNA from the vastus lateralis muscle was isolated using the Trizol method according to the manufacturer's protocol (Invitrogen, Merelbeke, Belgium).

Target mRNA	Accession nr.	PCR primer sequence 5' → 3'
MAFbx	NM_058229	F: GTG GTA CTG AAA GTC CTT GAA GAC R: TTA ATG TTC CCG ACC AGC A
MuRF1	NM_032588	F: GAA TAA CTG TAT CTC CAT GCT GG R: GGC ATA CAA CGT GTC AAA CTT
ATP5G2	NM_005175	F: GGA GAT ACT GAC AGA TGA GAG CC R: AAT GAA CTT GGC TGC TGT GTC
PET112L	NM_004564	F: GTG AGA GTC CTG TCA CAC CCT C R: CCT GTT TAG CTG CTG ATG AAG A
COX6C	NM_004374	F: CAG CTT TGT ATA AGT TTC GTG TGG R: ACC AGC CTT CCT CAT CTC CT
FoxO1A	NM_002015	F: CAA GAG CGT GCC CTA CTT CAA G R: CTG TTG TTG TCC ATG GAT GCA
FoxO3A	NM_001455	F: CTT CAA GCA TAA GGG CGA CA R: TCT TGC CAG TTC CCT CAT T

Table 3. Sequence of primers used for the real time PCR experiments. F: forward primer; R: reverse primer.

Microarray data acquisition and preprocessing. Total RNA was hybrized to a CodeLink UniSet Human 20K Bioarray containing 20,000 human transcripts (Amersham Biosciences, Diegem, Belgium) according to the manufacturer's protocol at the VIB MicroArray Facility (MAF, Leuven, Belgium). In brief, biotin-labelled cRNA was synthesized by i*n vitro* transcription and quality checked with the Nanodrop. Ten µg of biotin-labelled cRNA was fragmented and hybridized on the microarray slides and subsequently scanned with an Agilent Scanner. Gene expression was measured with the Codelink Expression Analysis software. All primary microarray data has been submitted in GEO under accession number GSE10828.

Microarray data analysis. Genemaths XT (Applied Maths, Belgium) was used for the analysis of the microarray data. Each microarray data file was background corrected and intensity values less than 1 were excluded from further analysis. Expression values were $\log_2$ transformed and quantiles normalized.

Because alterations in gene expression might manifest at the level of biological pathways or co-regulated gene sets rather than individual genes [16], gene ontology analysis and gene set enrichment analysis were used to analyze the microarray data.

The gene ontology project classifies genes into a hierarchy placing gene products with similar functions together. Because gene ontology is hierarchical, a gene that is in one category is automatically part of its parent classifications. Three structured controlled vocabularies (ontologies) describe gene products in terms of their associated biological processes, cellular components and molecular functions. The web-based applications GeneTrail [17] and DAVID (Database for annotation, visualization and integrated discovery) [18, 19] were used to analyze gene ontology enrichments in the differentially expressed transcripts. Transcripts with a $p<0.05$ were considered as differentially expressed and used as input for Ontology Analysis.

The gene set enrichment analysis (GSEA) represents a method of ranking pathways, or more generally gene sets, in terms of coupling (e.g. causing or being caused by) to a biological condition [16]. In contrast to Ontology analysis, the

Accession number	Description	P Value	ratio
NM_030754	serum amyloid A2	0.01526	5.44
NM_000331	serum amyloid A1	0.00077	4.60
NM_003064	secretory leukocyte protease inhibitor (SLPI)	0.00019	4.58
NM_002737	protein kinase C. alpha (PRKCA)	0.00237	4.57
NM_001657	amphiregulin (AREG)	0.00284	4.39
NM_015900	phosphatidylserine-specific phospholipase A1alpha (PS-PLA1)	0.00595	4.12
M81349	serum amyloid A	0.00094	4.04
NM_002422	matrix metalloproteinase 3 (MMP3)	0.00007	3.93
NM_002627	phosphofructokinase	0.00013	3.78
NM_016358	iroquois homeobox protein 4 (IRX4)	0.01207	3.77
NM_006398	ubiquitin D (UBD)	0.01133	3.71
NM_024111	hypothetical protein MGC4504	0.00359	3.66
NM_032762	hypothetical protein MGC16121	0.00218	3.57
NM_012108	BCR downstream signaling 1 (BRDG1)	0.04075	3.33
NM_030674	amino acid transporter system A1 (ATA1)	0.00008	3.14
NM_032898	hypothetical protein MGC14126	0.00154	3.09
NM_021817	brain link protein-1 (BRAL1)	0.03997	3.06
AF392452	oxysterol-binding protein-like protein (OSBPL8)	0.02078	2.90
NM_005218	defensin. beta 1 (DEFB1)	0.02623	2.85
NM_030751	transcription factor 8(TCF8)	0.00216	2.77
NM_013402	fatty acid desaturase 1 (FADS1)	0.01342	2.63
M22538	nuclear-encoded mitochondrial NADH-ubiquinone reductase 24Kd subunit	0.00068	2.61
AK026035	cDNA: FLJ22382 fis. clone HRC07514	0.01355	2.60
NM_016084	RAS. dexamethasone-induced 1 (RASD1)	0.01650	2.60
NM_002467	v-myc myelocytomatosis viral oncogene homolog (MYC)	0.02091	2.59

Table 4. Top 25 of the most highly up-regulated genes between COPD patients with acute exacerbation vs stable COPD patients.

Accession number	Description	P Value	ratio
AK026784	cDNA: FLJ23131 fis	0.02158	-3.94
410735.2	Protein with high similarity to Gremlin (human CKTSF1B1)	0.01112	-3.65
AF109718	chromosome 3 subtelomeric region	0.01558	-3.26
M31774	Human thyrotropin receptor (TSH)	0.00039	-2.96
U07563	proto-oncogene tyrosine-protein kinase (ABL) gene	0.02833	-2.88
BG913926	602812451F1	0.01636	-2.88
NM_018470	uncharacterized hypothalamus protein HT009 (HT009)	0.00010	-2.75
NM_024022	transmembrane protease. serine 3 (TMPRSS3)	0.01959	-2.68
NM_033424	myosin heavy chain-like (LOC92771)	0.02614	-2.66
NM_003014	secreted frizzled-related protein 4 (SFRP4)	0.01094	-2.64
NM_144583	hypothetical protein MGC20253 (MGC20253)	0.03529	-2.63
AK026659	cDNA: FLJ23006 fis	0.00195	-2.58
AB040945	KIAA1512	0.01627	-2.47
AK001663	cDNA FLJ10801 fis	0.01147	-2.41
NM_024087	DKFZP564L0862	0.04246	-2.24
D63412	aquaporin	0.00128	-2.22
AL137761	cDNA DKFZp586L2424	0.04040	-2.21
NM_004772	P311 protein (P311)	0.01332	-2.10
NM_021097	solute carrier family 8 (sodium/calcium exchanger). member 1 (SLC8A1)	0.02077	-2.07
BM723478	UI-E-EJ0-aio-p-06-0-UI.r1	0.00012	-2.06
NM_015133	mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3)	0.00531	-2.05
NM_003385	visinin-like 1 (VSNL1)	0.00204	-2.04
NM_152376	hypothetical protein FLJ25429	0.00038	-1.99
NM_001215	carbonic anhydrase VI (CA6)	0.02366	-1.98
AF147385	cDNA clone YI43B12	0.00301	-1.97

Table 5. Top 25 of the most highly down-regulated genes between COPD patients with acute exacerbation vs stable COPD patients.

GSEA algorithm is based on the usage of all available gene expression data. Data permutation is used to adjust for multiple testing. Gene Sets with a $p<0.05$ and a False Discovery Rate < 25%, cut off values as previously recommended [16], were assumed to be significantly enriched.

Finally, multidimensional scaling and principal component analysis were used as qualitative methodology to characterize the behavior of hospitalized and stable COPD patients. The analysis is based on the set genes differentially expressed between hospitalized and stable COPD patients.

	Number of genes	p values
Biological process		
Upregulated		
Ubiquitin-dependent protein catabolic process	19	0.04
Induction of apoptosis	22	0.03
Anti-apoptosis	24	0.04
Response to reactive oxygen species		0.04
Downregulated		
Aspartate Catabolism	3	0.03
Molecular function		
Downregulated		
Oxidoreductase activity acting on NADH or NADPH	13	0.04
Cellular compartment		
Downregulated		
Mitochondrial respiratory chain	16	0.02

Table 6. Gene Ontology enrichment in *vastus lateralis* of hospitalized vs. stable COPD patients. See text for details.

Quantitative real time polymerase chain reaction validation. First-strand cDNA was generated by reverse transcription using random primers and the Super-Script™ II kit (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. Amplification with primers (Table 3) was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the Platinum SYBR Green qPCR SuperMix UDG kit (Invitrogen). Copy numbers of unknown samples were calculated from plasmid cDNA standards. Data from each gene was expressed as copy number of 18S.

Statistical and Expression Analysis

Unpaired t-test was used to assess differences between patient characteristics. Data were expressed as mean and standard deviation.

For the microarray, statistical analysis was done with the Welsh t-test and corrected for multiple testing with Benjamini-Hoghbergs.

Mann Whitney test was used to compare gene amplification data obtained during real time PCR. Data were expressed as median and interquartile range.

Statistics were performed with the Statistical Analysis System software (SAS Institute Inc., North Carolina, USA). $P<0.05$ were considered significant.

Results

Patient characteristics (Table 1 and 2)

Hospitalized and stable COPD patients had moderate to severe airflow obstruction. Patients with an acute exacerbation had significantly higher CRP levels at admission and lower quadriceps muscle force compared to stable COPD patients. In the patients with an acute exacerbation, quadriceps force decreases on average by 4.4±7% during hospitalization.

Pathway	Number of genes	p-value	FDR
Upregulated transcripts			
Proteasomal degradation	21	0.05	0.10
Akt/FOXO	11	0.04	0.03
Downregulated transcripts			
Oxidative phosphorylation	61	0.04	0.21

Table 7. GSEA analysis showing enriched pathways in the vastus lateralis muscle of hospitalized compared to stable COPD patients. See text for details. FDR = False Discovery Rate.

Microarray data

Single gene microarray analysis revealed that 1,989 genes were differentially expressed between hospitalized and stable COPD patients, of which 930 were upregulated and 1,059 were downregulated. The top 25 of the most highly upregulated and downregulated genes are provided in table 4 and 5, respectively.

The multidimensional scaling plot of the principal component analysis reveals that hospitalized COPD patients represent a distinct population from stable COPD patients according to their gene expression.

Gene Ontology analysis (Table 6) revealed that several transcripts of the ubiquitin-dependent protein catabolism such as the ubiquitination factor 4A and the proteasome 26S subunit 5 were upregulated in hospitalized compared to stable COPD patients. Also transcripts of apoptosis and anti-apoptosis induction (TNF receptor-associated factor 3 and CASP8- and FADD-like apoptosis regulator) were upregulated as were transcripts of the response to reactive oxygen species namely superoxide dismutase 2 and glutathione peroxidase 3. In addition, analysis shows that transcripts of the aspartate catabolism, specifically the aspartate aminotransferase and the

		Number of genes	p value	FDR
Functional Group 1	Enrichment score: 6			
Category	Term			
GOTERM BP ALL	GO:0008219 cell death	94	$2.5x10^{-09}$	$4.9x10^{-08}$
GOTERM BP ALL	GO:0016265 death	94	$2.5x10^{-09}$	$4.9x10^{-08}$
GOTERM BP ALL	GO:0048468 cell development	125	$5.8x10^{-09}$	$1.1x10^{-07}$
GOTERM BP ALL	GO:0012501 programmed cell death	88	$1.7x10^{-08}$	$3.2x10^{-07}$
GOTERM BP ALL	GO:0006915 apoptosis	87	$2.3x10^{-08}$	$4.4x10^{-07}$
GOTERM BP ALL	GO:0043067 regulation of programmed cell death	60	$5.7x10^{-06}$	$1.1x10^{-04}$
GOTERM BP ALL	GO:0030154 cell differentiation	154	$7.5x10^{-06}$	$1.4x10^{-04}$
GOTERM BP ALL	GO:0048869 cellular developmental process	154	$7.5x10^{-06}$	$1.4x10^{-04}$
GOTERM BP ALL	GO:0042981 regulation of apoptosis	59	$8.1x10^{-06}$	$1.6x10^{-04}$
GOTERM BP ALL	GO:0043069 negative regulation of programmed cell death	32	$2.3x10^{-05}$	$4.3x10^{-04}$
GOTERM BP ALL	GO:0043066 negative regulation of apoptosis	31	$4.5x10^{-05}$	$8.5x10^{-04}$
Functional Group 2	Enrichment score: 2.85			
Category	Term			
GOTERM BP ALL	GO:0006950 response to stress	107	$1.9x10^{-07}$	$3.6x10^{-04}$
GOTERM BP ALL	GO:0009611 response to wounding	43	$1.2x10^{-03}$	0.22

Table 8. David analysis showing the genes being upregulated in the vastus lateralis muscle of hospitalized compared to stable COPD patients. FDR: False Discovery Rate.

		Number of genes	p value	FDR
Functional Group 1	Enrichment score: 2.31			
Category	Term			
GOTERM CC ALL	GO:0031980 mitochondrial lumen	33	$5.1x10^{-07}$	$7.9x10^{-06}$
GOTERM CC ALL	GO:0005759 mitochondrial matrix	33	$5.1x10^{-07}$	$7.9x10^{-06}$
GOTERM CC ALL	GO:0031974 membrane-enclosed lumen	95	$5.5x10^{-04}$	$8.5x10^{-03}$
GOTERM CC ALL	GO:0043233 organelle lumen	95	$5.5x10^{-04}$	$8.5x10^{-03}$
Functional Group 2	Enrichment score: 2.14			
Category	Term			
GOTERM BP ALL	GO:0006533 aspartate catabolic process	4	$1.4x10^{-03}$	$2.6x10^{-02}$
GOTERM BP ALL	GO:0006531 aspartate metabolic process	4	$1.4x10^{-03}$	$2.6x10^{-02}$
KEGG PATHWAY	hsa00252: Alanine and aspartate metabolism	10	$4.5x10^{-03}$	$5.9x10^{-02}$
GOTERM BP ALL	GO:0009068 aspartate family amino acid catabolic process	4	$1.0x10^{-02}$	0.18
Functional Group 3	Enrichment score: 1.94			
Category	Term			
GOTERM CC ALL	GO:0044429 mitochondrial part	65	$1.9x10^{-05}$	$3.1x10^{-04}$
GOTERM CC ALL	GO:0031967 organelle envelope	57	$7.4x10^{-03}$	0.11
GOTERM CC ALL	GO:0031975 envelope	57	$7.9x10^{-03}$	0.12
GOTERM CC ALL	GO:0044455 mitochondrial membrane part	16	$1.8x10^{-02}$	0.24
Functional Group 4	Enrichment score: 1.23			
Category	Term			
GOTERM BP ALL	GO:0006119 oxidative phosphorylation	17	$7.6x10^{-03}$	0.13
GOTERM CC ALL	GO:0044455 mitochondrial membrane part	16	$1.8x10^{-02}$	0.24

Table 9. David analysis showing the genes being downregulated in the vastus lateralis muscle of hospitalized compared to stable COPD patients. FDR: False Discovery Rate.

aspartoacyclase were downregulated in hospitalized COPD patients. This was also the case for the two related ontologies mitochondrial respiratory chain and oxidoreductase activity acting on NADH or NADPH, such as the cytochrome c oxidase subunit 6C and the NADH-ubiquinone oxidoreductase Fe-S protein 1.

According to the GSEA algorithm, transcripts belonging to proteasomal degradation and the Akt/FoxO pathways were upregulated while transcripts belonging to the oxidative phosphorylation pathway were repressed in the vastus lateralis of hospitalized patients compared to stable COPD patients (Table 7).

As shown on table 8 and 9, DAVID analysis confirmed these data although the upregulation of the ubiquitin-dependent protein catabolism was not found with this analysis.

Real-time PCR data

The proteasomal degradation and Akt/FoxO pathways as well as the oxidative phosphorylation pathways were selected for further analysis. MuRF-1 and MAFbx were chosen as key-genes of the proteasomal degradation pathway. They are both coding for muscle specific atrophy-related ubiquitin protein ligases. MuRF-1 and MAFbx mRNA was significantly higher in hospitalized patients compared to stable COPD patients (Fig. 1A, and 1B). A 1.5 fold change in FoxO1A mRNA was found in the hospitalized patients compared to stable COPD patients while a -4.5 fold changes were found in FoxO3A mRNA. However, these data did not reach statistical significance.

COX6C was chosen as marker of the oxidative phosphorylation pathway as it is coding for a subunit of cytochrome c oxidase. COX6C mRNA was significantly lower in hospitalized compared to stable COPD patients (Fig. 1C). Similarly, mRNA levels of ATP5G2 (encoding a subunit of mitochondrial ATP synthase) and PET112L (involved in formation of correctly charged Gln-tRNA) were downregulated (fold changes: -5.5 and -4.9, respectively) in hospitalized compared to stable COPD patients although these alterations failed to reach statistical significance.

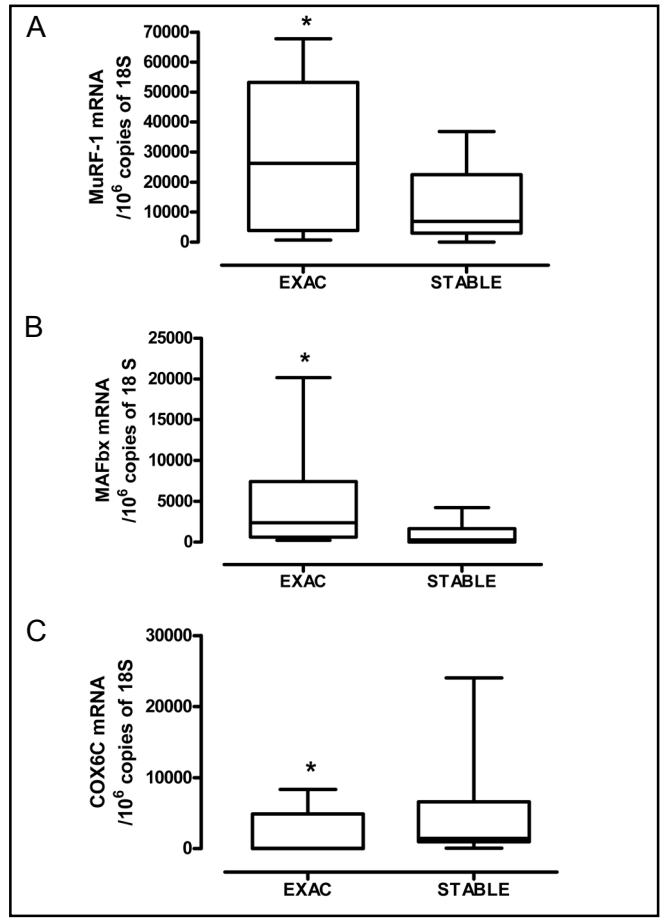

Fig. 1. mRNA expression levels of MURF-1 (A), MAFbx (B) and COX6C (C) in the vastus lateralis muscle of COPD patients with an acute exacerbation (EXAC) and stable COPD patients (STABLE). Data are expressed as copy number per million copies of 18S and are presented as median and interquartile range. * $p < 0.05$.

Discussion

The present study showed that in the vastus lateralis muscle of COPD patients with an acute exacerbation, the ubiquitin-dependent protein catabolism, the Akt/FoxO pathway, the induction of apoptosis and anti-apoptosis and the response to reactive oxygen species are upregulated while the aspartate catabolism, the oxido reductase activity acting on NADP-NADPH, the oxidative phosphorylation and the mitochondrial respiratory chain are downregulated. Real-time PCR showing increased MuRF1 and MAFbx mRNA and decreased COX6C mRNA confirmed the activation of the ubiquitin-dependent protein catabolism and the downregulation of mitochondrial respiration pathway.

This study is the first showing that pathways leading to muscle atrophy are activated in the muscle of COPD patients during an acute exacerbation. In particular the ubiquitin-proteasome pathway, the Akt/FoxO pathway but also molecular triggers (reactive oxygen species, apoptosis process) are upregulated during exacerbation. In fact, most of these pathways or processes are already activated in the muscle of stable COPD patients.

Indeed, a micro-array study has already reported activation of the ubiquitin-proteasome pathway in the quadriceps muscle of stable COPD patients compared to controls [10]. Activation of this pathway is further evidenced by the increased MuRF1 mRNA and MAFbx mRNA and protein levels in the quadriceps muscle of stable COPD patients presenting muscle atrophy [20]. In these patients, the cytoplasmatic protein content of the phosphorylated form of Akt was also elevated [20] and FoxO-1 and to a lesser extent FoxO-3 were upregulated [9, 20]. As expected, protein breakdown is increased in stable COPD patients with low BMI [21]. The data of the present study revealed that these processes are more pronounced in the muscle of COPD patients suffering from an acute exacerbation. This is particularly important as this may result in enhanced proteolysis leading to a further decrease in muscle mass and evidently having an

impact on muscle function. In fact, these processes are closely interrelated. The Akt/FoxO pathway is able to modulate the expression of MuRF1 and MAFbx [22]. In the absence of IGF-I, the FoxO transcription factors are dephosphorylated and translocated to the nucleus to drive the expression of MuRF1 and MAFbx. This is particularly interesting knowing that muscle IGF-I expression is decreased in COPD patients with an acute exacerbation [15]. This suggests that activation of the ubiquitin-proteasome pathway via the FoxO pathway develops in the muscle of COPD patients during an acute exacerbation.

Increase in reactive oxygen species is thought to reflect damage caused by oxidative stress. Higher expression of genes involved in oxidative stress has been reported in the quadriceps muscle of stable COPD patients compared to controls [10]. In addition, increased levels of lipid peroxidation [23], protein carbonylation [24] and hydrogen peroxide [25] have all been reported in the vastus lateralis muscle of stable COPD at rest. More recently, direct measurement of ROS production in mitochondria isolated from the vastus lateralis muscle of moderate COPD patients showed that ROS are enhanced at rest in these patients [26]. This means that the oxidative stress which is already present in the muscle of stable COPD patients is further exaggerated during an acute exacerbation as shown in the present study. Excessive ROS production within the muscle is known to mainly target mitochondria and myofilaments. This would lead to apoptosis, mitochondrial respiratory chain dysfunction and/or alteration in myofilament contractile properties (see [27] for a review). This is in agreement with the data of the present study suggesting upregulation of apoptosis and downregulation of mitochondrial respiration process.

The presence of increased apoptosis namely higher percentage of apoptotic myonuclei has been reported in the quadriceps muscle of stable COPD with low BMI in whom BMI was inversely related to skeletal muscle apoptosis [28]. In the present study, apoptosis was further activated during an acute exacerbation. Although apoptosis leads normally to cell death, it can also cause cell atrophy especially in multinucleated cells [29]. In skeletal muscle fibers, each myonucleus regulates the gene products and protein expression in a limited volume of the muscle fiber. This volume is termed the myonuclear domain. Decrease in myonuclear domain through apoptosis has been reported in several experimental models of muscle atrophy [30]. Also in patients with heart failure, apoptosis, while measuring the amount of apoptotic nuclei, increased in the vastus lateralis muscle and the magnitude of apoptosis was associated with the degree of muscle atrophy [31]. It has, therefore, been proposed that apoptosis might play a role in determining muscle bulk loss [31]. If so, this would suggest that apoptosis developing in the muscle of COPD patients during an acute exacerbation would probably contribute to muscle wasting. The latter may affect muscle force and may eventually postpone muscle recovery in these patients.

The two main signaling pathways of apoptosis are mediated either through the death receptor family or through the mitochondrial apoptotic pathway. In the latter, apoptosis is initiated by the release of cytochrome c from the mitochondria which forms a complex with the apoptotic protease activating factor-1 and the pro-caspase-9 leading to the activation of caspase-9 and caspase-3. Basal amount of cytochrome c was recently shown to be elevated in different muscles of stable COPD patients [26]. In addition, increased levels of cytochrome oxidase which uses cytochrome c as substrate have been found in the quadriceps of these patients [32]. Importantly, this mitochondrial apoptotic pathway is stimulated by reactive oxygen species [33] and those are upregulated in the muscle of COPD patients during an acute exacerbation. Furthermore, IGF-I that is known to eventually exert anti-apoptotic effects while inhibiting the pro-apoptotic factor, *bad*, or the caspase-9 [33] is decreased in serum and muscle of COPD patients during an acute exacerbation [14, 15]. This underlines that the role of apoptosis during an acute exacerbation of COPD should not be neglected.

The present study also revealed that the expression of several pathways was downregulated during exacerbation. Those pathways involved the aspartate catabolism, the oxido reductase activity acting on NADP-NADPH, the oxidative phosphorylation and the mitochondrial respiratory chain. In fact, these observations pointed out that the mitochondrial function is altered since all these processes are belonging to the mitochondria system. Indeed, the mitochondrial chain is a set of metabolic reactions and processes that take place to convert biochemical energy from nutrients into adenosine triphosphatase (ATP). Transfer of electron from one redox reaction to another is catalyzed by oxidoreductases. Oxidative phosphorylation is the metabolic pathway using energy released by the oxidation of nutrients to produce ATP. Aspartate carries reducing equivalents in the malate-aspartate shuttle. All these processes are altered during an acute exacerbation. This means that ATP production is impaired.

Evidence for abnormal mitochondrial function was already described in the muscle of stable COPD patients.

Oxidative phosphorylation efficiency was decreased in the vastus lateralis muscle of stable COPD patients, and this was even worse in those with low BMI [34]. In addition, protein levels of uncoupling protein-3 (UCP-3) were shown to be reduced in the vastus lateralis muscle of stable COPD patients [35]. This protein, predominantly expressed in skeletal muscle, uncouples oxidative phosphorylation from ATP production [36]. It has been suggested that UCP-3 could play a role in limiting ROS production since mitochondria from skeletal muscle of UCP3-deficient mice show an increased ROS production [37].

The consequences of this abnormal mitochondrial function are obvious for the muscles. The end result of mitochondrial dysfunction is the impaired aerobic ATP production. ATP provides the immediate source of energy for muscle contraction and for pumping ions to maintain the muscle fiber plasmalemma in an excitable state during muscle activity. ATP is also required for many of the usual muscle functions during and between periods of activity. Even for relatively brief periods of activity, the muscle must be able to renew its supply of ATP. In COPD patients, impaired muscle function is partly due to mitochondrial dysfunction as described previously. In addition, the present study suggests that the alterations in mitochondrial function already present in stable COPD patients are worsened during an acute exacerbation. This may contribute to the further deterioration of muscle function seen during exacerbation and this may compromise recovery after exacerbation. It is therefore not surprising that COPD patients after an exacerbation are weak and inactive. Attention to minimize impaired mitochondrial function during exacerbation should be considered.

It is clear that the alterations seen in the muscle of COPD patients during an acute exacerbation have been triggered by several factors. Among them, inflammation related to exacerbation, undernutrition, muscle inactivity during bed rest and use of systemic corticosteroid are probably playing an important role. These conditions are all known to affect the above-mentioned pathways that are altered during an acute exacerbation. Therefore, one should consider developing strategies to minimize the loss in muscle function occurring during an exacerbation. This could be achieved either while targeting the triggering factors or by acting on key elements of the altered pathways. This is particularly important knowing that muscle function recovers very slowly after an exacerbation. In some COPD patients with repeated exacerbations, muscle function may therefore be severely impaired especially if exacerbation occurs when muscle did not yet recover from the previous exacerbation.

In conclusion, the present study suggests that several pathways leading to muscle atrophy and mitochondrial dysfunction are activated in the muscle of COPD patients during an acute exacerbation. These alterations potentially contribute to loss in muscle force described in COPD patients during an acute exacerbation and would probably lead to muscle wasting. Therefore, therapeutic interventions should be designed to counteract the detrimental effect of exacerbation on these pathways.

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References

1 Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, Fukuchi Y, Jenkins C, Rodriguez-Roisin R, van Weel C, Zielinski J: Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 2007;176:532-555.

2 Agusti AG, Noguera A, Sauleda J, Sala E, Pons J, Busquets X: Systemic effects of chronic obstructive pulmonary disease. Eur Respir J 2003;21:347-360.

3 Pitta F, Troosters T, Spruit MA, Probst VS, Decramer M, Gosselink R: Characteristics of physical activities in daily life in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2005;171:972-977.

4 Swallow EB, Reyes D, Hopkinson NS, Man WD, Porcher R, Cetti EJ, Moore AJ, Moxham J, Polkey MI: Quadriceps strength predicts mortality in patients with moderate to severe chronic obstructive pulmonary disease. Thorax 2007;62:115-120.

5 Pitta F, Troosters T, Spruit MA, Decramer M, Gosselink R: Activity monitoring for assessment of physical activities in daily life in patients with chronic obstructive pulmonary disease. Arch Phys Med Rehabil 2005;86:1979-1985.
6 Decramer M, Lacquet LM, Fagard R, Rogiers P: Corticosteroids contribute to muscle weakness in chronic airflow obstruction. Am J Respir Crit Care Med 1994;150:11-16.
7 Debigare R, Cote CH, Maltais F: Peripheral muscle wasting in chronic obstructive pulmonary disease. Clinical relevance and mechanisms. Am J Respir Crit Care Med 2001;164:1712-1717.
8 Couillard A, Maltais F, Saey D, Debigare R, Michaud A, Koechlin C, LeBlanc P, Prefaut C: Exercise-induced quadriceps oxidative stress and peripheral muscle dysfunction in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2003;167:1664-1669.
9 Debigare R, Maltais F, Cote CH, Michaud A, Caron MA, Mofarrahi M, LeBlanc P, Hussain SN: Profiling of mRNA expression in quadriceps of patients with COPD and muscle wasting. COPD 2008;5:75-84.
10 Radom-Aizik S, Kaminski N, Hayek S, Halkin H, Cooper DM, Ben-Dov I: Effects of exercise training on quadriceps muscle gene expression in chronic obstructive pulmonary disease. J Appl Physiol 2007;102:1976-1984.
11 Donaldson GC, Seemungal TA, Bhowmik A, Wedzicha JA: Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. Thorax 2002;57:847-852.
12 Seemungal TA, Donaldson GC, Paul EA, Bestall JC, Jeffries DJ, Wedzicha JA: Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1998;157:1418-1422.
13 Soler-Cataluna JJ, Martinez-Garcia MA, Roman SP, Salcedo E, Navarro M, Ochando R: Severe acute exacerbations and mortality in patients with chronic obstructive pulmonary disease. Thorax 2005;60:925-931.
14 Spruit MA, Gosselink R, Troosters T, Kasran A, Gayan-Ramirez G, Bogaerts P, Bouillon R, Decramer M: Muscle force during an acute exacerbation in hospitalised patients with COPD and its relationship with CXCL8 and IGF-I. Thorax 2003;58:752-756.
15 Crul T, Spruit MA, Gayan-Ramirez G, Quarck R, Gosselink R, Troosters T, Pitta F, Decramer M: Markers of inflammation and disuse in vastus lateralis of chronic obstructive pulmonary disease patients. Eur J Clin Invest 2007;37:897-904.
16 Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP: Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-15550.
17 Backes C, Keller A, Kuentzer J, Kneissl B, Comtesse N, Elnakady YA, Muller R, Meese E, Lenhof HP: GeneTrail—advanced gene set enrichment analysis. Nucleic Acids Res 2007;35:W186-W192.
18 Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA: DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 2003;4:3.
19 Huang da W, Sherman BT, Lempicki RA: Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44-57.
20 Doucet M, Russell AP, Leger B, Debigare R, Joanisse DR, Caron MA, LeBlanc P, Maltais F: Muscle Atrophy and Hypertrophy Signalling in Patients with Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2007;176:261-269.
21 Rutten EP, Franssen FM, Engelen MP, Wouters EF, Deutz NE, Schols AM: Greater whole-body myofibrillar protein breakdown in cachectic patients with chronic obstructive pulmonary disease. Am J Clin Nutr 2006;83:829-834.
22 Glass DJ: Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol 2005;37:1974-1984.
23 Barreiro E, de la Puente B, Minguella J, Corominas JM, Serrano S, Hussain SN, Gea J: Oxidative stress and respiratory muscle dysfunction in severe chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2005;171:1116-1124.
24 Barreiro E, Schols AM, Polkey MI, Galdiz JB, Gosker HR, Swallow EB, Coronell C, Gea J: Cytokine profile in quadriceps muscles of patients with severe chronic obstructive pulmonary disease. Thorax 2008;63:100-107.
25 Picard M, Godin R, Sinnreich M, Baril J, Bourbeau J, Perrault H, Taivassalo T, Burelle Y: The mitochondrial phenotype of peripheral muscle in chronic obstructive pulmonary disease: disuse or dysfunction? Am J Respir Crit Care Med 2008;178:1040-1047.
26 Puente-Maestu L, Perez-Parra J, Godoy R, Moreno N, Tejedor A, Gonzalez-Aragoneses F, Bravo JL, Villar F, Camano S, Agusti A: Abnormal mitochondrial function in locomotor and respiratory muscles of COPD patients. Eur Respir J 2009;33:1045-1052.
27 Couillard A, Prefaut C: From muscle disuse to myopathy in COPD: potential contribution of oxidative stress. Eur Respir J 2005;26:703-719.
28 Agusti AGN, Sauleda J, Miralles C, Gomez C, Togores B, Sala E, Batle S, Busquets S: Skeletal muscle apoptosis and weight loss in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2002;166:485-489.
29 Allen DL, Linderman JK, Roy RR, Bigbee AJ, Grindeland RE, Mukku V, Edgerton VR: Apoptosis: a mechanism contributing to remodeling of skeletal muscle in response to hindlimb unweighting. Am J Physiol 1997;273:C579-C587.
30 Allen DL, Roy RR, Edgerton VR: Myonuclear domains in muscle adaptation and disease. Muscle Nerve 1999;22:1350-1360.
31 Vescovo G, Volterrani M, Zennaro R, Sandri M, Ceconi C, Lorusso R, Ferrari R, Ambrosio GB, Dalla LL: Apoptosis in the skeletal muscle of patients with heart failure: investigation of clinical and biochemical changes. Heart 2000;84:431-437.
32 Sauleda J, Garcia-Palmer F, Wiesner RJ, Tarraga S, Harting I, Tomas P, Gomez C, Saus C, Palou A, Agusti AG: Cytochrome oxidase activity and mitochondrial gene expression in skeletal muscle of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1998;157:1413-1417.
33 Lewis MI: Apoptosis as a potential mechanism of muscle cachexia in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2002;166:434-436.
34 Rabinovich RA, Bastos R, Ardite E, Llinas L, Orozco-Levi M, Gea J, Vilaro J, Barbera JA, Rodriguez-Roisin R, Fernandez-Checa JC, Roca J: Mitochondrial dysfunction in COPD patients with low body mass index. Eur Respir J 2007;29:643-650.
35 Gosker HR, Schrauwen P, Hesselink MK, Schaart G, van der Vusse GJ, Wouters EF, Schols AM: Uncoupling protein-3 content is decreased in peripheral skeletal muscle of patients with COPD. Eur Respir J 2003;22:88-93.
36 Boss O, Samec S, Paoloni-Giacobino A, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino JP: Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. FEBS Lett 1997;408:39-42.
37 Vidal-Puig AJ, Grujic D, Zhang CY, Hagen T, Boss O, Ido Y, Szczepanik A, Wade J, Mootha V, Cortright R, Muoio DM, Lowell BB: Energy metabolism in uncoupling protein 3 gene knockout mice. J Biol Chem 2000;275:16258-16266.